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## METHODOLOGY

# Comparison of two different culture conditions for derivation of early hiPSC

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## Abstract

Different culture-systems for derivation of induced pluripotent stem cells (iPSC) in vitro from human fibroblasts have been established. Here, we compared the efficacy of two different feeder-free culture-systems; Matrigel-coated surfaces in combination with mTeSR1 medium versus Vitronectin-coated surfaces in combination with Essential 8 (E8) medium. The comparison was performed by counting the number of emerging iPSC-looking colonies of re-programmed fibroblasts. The fibroblasts were re-programmed using episomal plasmids expressing *OCT3/4*, *SOX2*, *KLF4*, *L-MYC*, *LIN28*, and a p53 knock down *shP53*. Three different fibroblast lines, K40 and K48 from healthy controls and BBS1 from a patient with Bardet–Biedl syndrome, were used in two independent setups. The BBS1 line was used in both setups in combination with K40 and K48 respectively. In all four re-programming experiments, we observed a significantly higher number of emerging colonies with the combination Matrigel/mTeSR1 as compared to the combination Vitronectin/E8. The presence of iPSC was verified by alkaline phosphatase and Tra-1-60 staining. Furthermore, a higher expression of the pluripotency-associated markers *NANOG* and *SOX2* in cells under Matrigel/mTeSR1 conditions compared with Vitronectin/E8 supported the higher proportion of iPSC on Matrigel/mTeSR1 plates. In conclusion, the combination Matrigel/mTeSR1 is more efficient for derivation of iPSC compared to the Vitronectin/E8 combination.

**Keywords:** E8; iPSC; mTeSR1; Matrigel; Vitronectin

## Background

The discovery of pluripotent stem cells in 1981 by Evans and Kaufman were thought to initiate a revolution in research and the treatment of diseases (Evans and Kaufman, 1981). They demonstrated that cells from the inner cell mass of mouse blastocysts were undifferentiated, and capable of developing into cells of all three germ layers; endoderm, mesoderm, and ectoderm (Evans and Kaufman, 1981; Martin, 1981). The use of embryos to obtain these cells however raised ethical concerns. This problem was recently resolved by the groundbreaking discovery of the ability to induce a new type of pluripotent stem cell from somatic cells; Takahashi and Yamanaka discovered in 2006 that mouse (Takahashi and Yamanaka, 2006) and in 2007 (Takahashi

et al., 2007) human fibroblast cells could be re-programmed into a pluripotent state by adding factors essential to embryonic development (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). After identifying 24 genes encoding pluripotency-inducing factors, they found only two of these to be essential for the re-programming process, and two to be dispensable but increasing the re-programming efficiency markedly (Takahashi and Yamanaka, 2006). This new type of stem cell is known as induced pluripotent stem cells (iPSC), and the factors they used are known as the “OSKM” factors; *OCT3/4*, *SOX2*, *KLF4*, and *C-MYC* (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). In 2007 another group also succeeded in generating human iPSC using *OCT4*, *SOX2*, *NANOG*, and *LIN28* (Yu et al., 2007). Successful generation of human iPSC subsequently allowed

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**Abbreviations:** AP, alkaline phosphatase; BSA, bovine serum albumin; C<sub>T</sub>, threshold cycle; E8, essential 8; EBNA-1, Epstein-Barr Nuclear antigen –1; ESC, embryonic stem cells; EHS, Engelbreth-Holm-Swarm; GMP, good manufacturing practice; iPSC, induced pluripotent stem cells

researchers to create disease-specific model systems and patient-specific iPSC, bringing cell therapy one step closer to reality (Rodríguez-Pizá *et al.*, 2010). Since the discovery of iPSC, focus has been directed towards optimizing re-programming efficiency and derivation of pluripotent stem cells (iPSC). This has been achieved by investigating additional re-programming factors (Yu *et al.*, 2007; Yu *et al.*, 2009) and how these factors are delivered to the cells as well as the chosen culture system. Optimizations used today include a brief suppression of p53 by adding a short hairpin RNA against p53 (shP53) (Hong *et al.*, 2009; Rasmussen *et al.*, 2014) and using *L-MYC* instead of *C-MYC* to increase the re-programming efficiency and to decrease the risk of tumorigenesis (Okita *et al.*, 2011).

There are several systems for the delivery of re-programming factors. Episomal plasmids are widely used today (Yu *et al.*, 2009; Okita *et al.*, 2011; Rasmussen *et al.*, 2014). This technique is based on the Epstein-Barr Nuclear Antigen-1 (oriP/EBNA-1) system that has been proven to generate iPSC without any transgenic sequences integrating into the target cell genome (Yu *et al.*, 2009). The oriP/EBNA-1 system replicates only once per cell cycle and has been reported to be lost over time without drug selection (Yates and Guan, 1991; Yu *et al.*, 2009).

Murine feeder cells and conditioned media were previously used routinely to support cell growth and maintain the pluripotency of embryonic stem cells (ESC) and iPSC. However, culture conditions have since been optimized and simplified to achieve a more standardized and reproducible result. Today, mTeSR1 and Essential 8 (E8), both based on DMEM-F12, are the two widely used stem cell-supporting media. The mTeSR1 medium is serum-free, but does not have a completely defined formulation because it contains bovine serum albumin (BSA) (Ludwig *et al.*, 2006) known to have batch variations which could affect the outcome. A simplified version of mTeSR1 was subsequently developed; the completely chemically defined E8. These media contain only 8 components, all synthetic, identified from 16 components included in mTeSR1 that are required and sufficient for cell-survival and the prevention of spontaneous differentiation of iPSC (Chen *et al.*, 2011).

Basement matrices have been developed and optimized for both media types. Matrigel<sup>®</sup> matrix (Corning) is a widely used product, but as it is extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells it is not completely chemically defined (Kleinman *et al.*, 1982; Kleinman *et al.*, 1986; Bissell *et al.*, 1987; McGuire and Seeds, 1989; Vukicevic *et al.*, 1992). To circumvent this, a completely synthetic and simplified basement matrix composed of Vitronectin was designed based on the clarification of adherence receptors used by human ESC (Chen *et al.*, 2011). Matrigel matrix is typically used in

combination with mTeSR1 and Vitronectin is typically used in combination with E8 media, and both conditions have been shown to sustain iPSC expansion for more than 10 passages (Ludwig *et al.*, 2006; Chen *et al.*, 2011).

Here, we compared the two commonly used feeder-free culture conditions for derivation of induced pluripotent stem cells (iPSC) at an early stage after reprogramming; Matrigel combined with mTeSR1 compared to Vitronectin combined with E8 (Okita *et al.*, 2011; Rasmussen *et al.*, 2014). Fibroblasts were reprogrammed using three episomal plasmids coding for a modified human version of the OSKM genes and a short hairpin RNA against p53 (shp53).

## Materials and methods

### Cell culture

Fibroblast cell cultures were established from skin biopsies. The fibroblasts were grown in T75 flasks (Sarstedt #2020-06) using DMEM F-12 + GlutaMAX (Gibco #31331-028) with 10% bovine calf serum and 1% pen/strep at 37°C in 5% CO<sub>2</sub> and passaged with trypsin-EDTA (Gibco #25300-054) when they reached confluency. Two different fibroblast-lines from healthy controls, K40 and K48, and one fibroblast-line from a patient with Bardet-Biedl syndrome, BBS1 were used.

### iPSC re-programming

iPSC re-programming of human dermal fibroblasts was performed as described previously (Okita *et al.*, 2011; Rasmussen *et al.*, 2014) by electroporation with episomal plasmids that encode *hSOX2* and *hKLF4* (addgene plasmid #27078) *hL-MYC* and *hLIN28* (addgene plasmid #27080) and *hOCT4* with a short hairpin RNA to *P53* (shp53) (addgene plasmid #27077). Each plasmid was used at an amount of 1.25 µg in the ratio 1:1:1. The plasmids were introduced with Amaxa Nucleofector<sup>™</sup> 2b (Lonza) using the Amaxa Basic Nucleofector Kit for Primary Mammalian Fibroblasts (Lonza #VPI-1002) following the manufacturer's protocol with program V-024;

[http://bio.lonza.com/fileadmin/groups/marketing/Downloads/Protocols/Generated/Optimized\\_Protocol\\_226.pdf](http://bio.lonza.com/fileadmin/groups/marketing/Downloads/Protocols/Generated/Optimized_Protocol_226.pdf).

Seven days after electroporation, the cells were split and transferred to six well plates (CellStar #657 160) coated with Corning Matrigel Matrix (Corning #354277) or Vitronectin XFTM (Stemcell #07180), respectively ( $6 \times 10^4$  cells/well). The coating was performed according to the manufacturer's instructions. The Matrigel-coated wells contained pre-warmed mTeSR1 (Stemcell #05857) medium while the Vitronectin-coated wells contained pre-warmed E8 medium (ThermoFischer #A1517001). The media were supplemented with 1% pen/strep. The cells were grown in 5% CO<sub>2</sub>, 5% O<sub>2</sub> in N<sub>2</sub> at 37°C.

### Staining for alkaline phosphatase

On Day 28 after re-programming, iPSC colonies were stained for alkaline phosphatase (AP) activity using the iPSC characterization kit, SAB-KIT-1 (System Biosciences). The iPSC were fixed for 5 min with 4% paraformaldehyde (PFA) and incubated for 20 min in the dark in 500  $\mu$ L of AP stain-mix composed of the two components “A” and “B” mixed 1:1 according to the manufacturer’s instructions. The cells were kept in PBS until microscopy.

### Staining for Tra-1-60

The cells were fixed for 15 min in 4% PFA followed by washing and incubation in permeabilization buffer (0.2% TritonX-100, in PBS) for 15 min and then in blocking buffer (3% bovine serum albumin (BSA) (Tocris Bioscience #5217)) diluted in permeabilization buffer for 30 min. The cells were incubated with the primary antibody (Tra-1-60 (System Biosciences, #SAB-100A-1, 1:100)), diluted in blocking buffer for 2 h at room temperature. This step was followed by a wash in blocking buffer and incubation with the secondary antibodies (ThermoFisher Scientific, #A10036, 1:800) diluted in blocking buffer at room temperature for 45 min. The cells were kept in PBS until microscopy.

### Quantitative real-time PCR

The cells were harvested using trypsin-EDTA (Gibco #25300-054), and total RNA was extracted using the Qiagen RNeasy Mini Kit (#74106) according to the manufacturer’s instructions. A NanoDrop ND-1000 spectrophotometer was used to measure the RNA concentrations. The High Capacity cDNA kit (Applied Biosystems #4368813) was used for the preparation of cDNA. The cDNA samples were assayed in triplicate in 16.7 ng/sample in a total volume of 25  $\mu$ L. Universal PCR Master Mix (Applied Biosystems #4318157) and Taqman probes against *SOX2* and *NANOG* transcripts (*SOX2*; hs01053049s1, *NANOG*; hs04260366g1 from Applied Biosystems) both spanning exons were used. A FAM labeled probe and primers for the human *GAPDH* transcript (part number 4352934E) were used as an endogenous control. A relative quantification of *GAPDH* transcript was carried out on parallel samples. PCR amplification and detection were performed with an ABI7500 (Applied Biosystems, www.invitrogen.com) in accordance with the manufacturer’s instructions. The  $C_T$  value is the cycle number at which the fluorescence generated within a reaction crosses the threshold line. Standard curves for  $C_T$  values compared with log cDNA concentration were prepared by assaying threefold serial dilutions of control cDNA: from 50 ng/sample to 0.62 ng/sample. The amount of transcript was calculated by linear regression of the lines generated by the standard curves, log cDNA concentration against  $C_T$  (<http://www3.appliedbiosystems.com/cms/groups/>

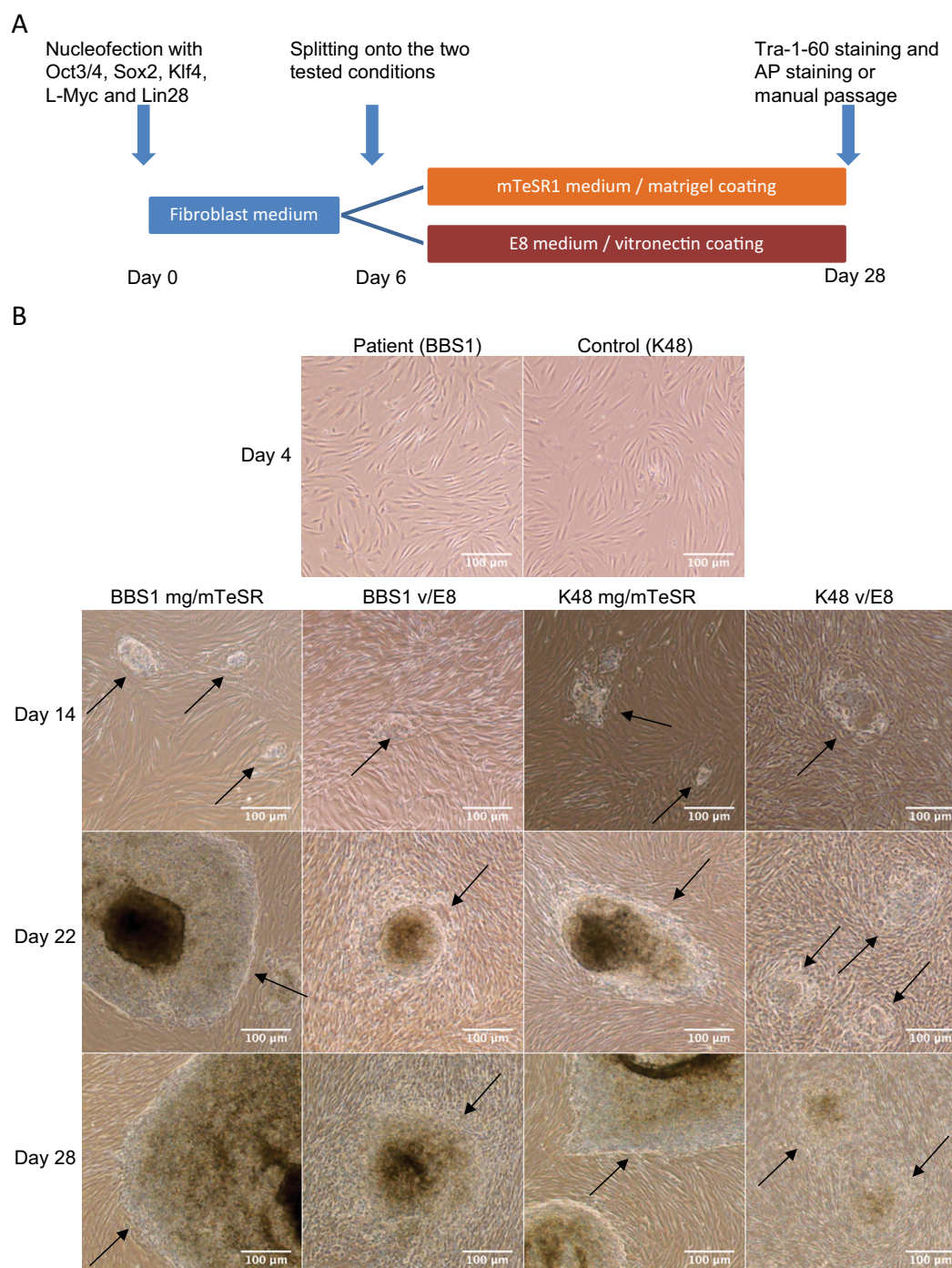
mcb\_support/documents/generaldocuments/cms\_042380.pdf). The normalized *SOX2* and *NANOG* values were calculated by dividing with the *GAPDH* value.

### Results and discussion

To ascertain that the obtained results were not cell-line specific, three different human dermal fibroblast cell lines were re-programmed. In brief, one primary fibroblast-line, BBS1 were obtained from a patient suffering from Bardet-Biedl syndrome, and two fibroblast-lines, K40, and K48 from two healthy controls. The re-programming was performed in two independent setups, with the inclusion of BBS1 in both setups. In one setup, BBS1 in combination with K48 were re-programmed (Figure 1), whereas BBS1 and K40 were re-programmed in another setup (Figure S1). The fibroblast-lines were re-programmed into iPSC by nucleofection of three episomal plasmids, encoding the human genes; *OCT3/4*, *SOX2*, *KLF4*, *L-MYC*, *LIN28*, and a p53 knock down *shP53* ( $5 \times 10^5$  cells of each type per transfection). Six days later, half of the cells were re-seeded on Matrigel-coated plates combined with mTeSR1 medium, whereas the other half were plated on Vitronectin-coated plates combined with E8 ( $6 \times 10^4$  cells/well) (Figures 1A and S1).

The morphological changes were monitored carefully during the 28 day course of the experiment. By Day 10–14 after electroporation, stem cell-looking colonies were easily visible (Figures 1B and S1). The size and morphology of the colonies on Matrigel/mTeSR1 plates were larger and more well-defined compared to the colonies that developed on Vitronectin/E8 plates. This tendency became more pronounced with time, after 22–23 days and 27–28 days, respectively (Figures 1B and S1). By Day 28, the colonies formed on Matrigel/mTeSR1 plates had grown large in size and began to show signs of differentiation in the middle. Colonies grown on Vitronectin/E8 plates also showed signs of differentiation but did not seem to increase in size to the same extent, and the overgrowth of surrounding cells continued, making the stem cell-looking colonies hard to distinguish from the surrounding fibroblasts or partially reprogrammed cells. Morphologically, the colonies obtained on the Matrigel/mTeSR1 plates were round in shape with well-defined edges, whereas the colonies on the Vitronectin/E8 plates were more diffuse, embedded in the surrounding cells and generally appeared to be smaller (Figures 1B and S1). There was no difference in the morphology or size of the colonies which had formed from the fibroblast control cell-lines, versus the colonies formed from the Bardet-Biedl patient fibroblast-line.

On Day 28, selected colonies from both the patient and healthy control grown under the two different conditions were investigated for their expression of the pluripotency markers alkaline phosphatase and Tra-1-60 (Yu *et al.*, 2007). All tested presumed stem-cell colonies stained positive for

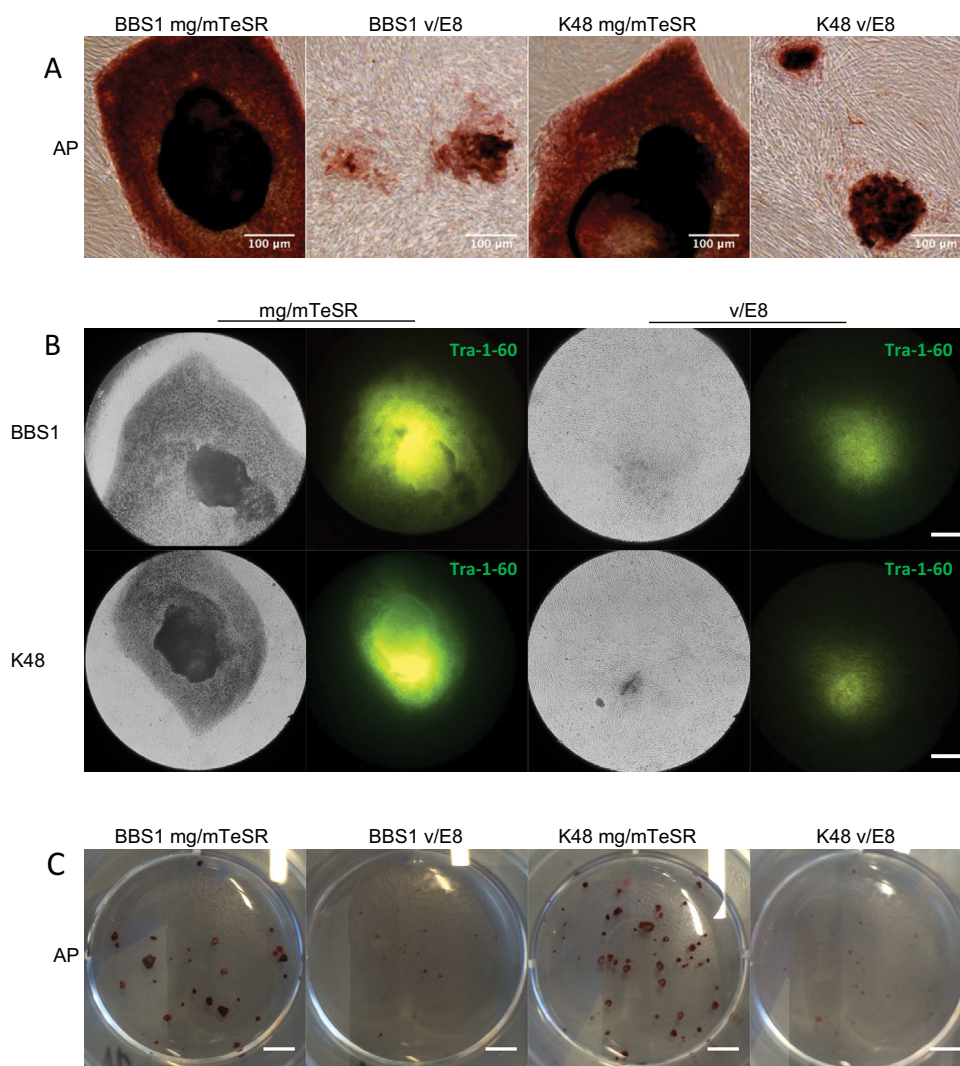


**Figure 1** Re-programming setup and observation of morphological changes during the course of re-programming. (A) Scheme depicting the protocol for fibroblast re-programming with the episomal plasmids expressing hOCT4, hSOX2, hKLF4, hL-MYC, hLIN28, and shp53 grown under Matrigel/mTeSR1 (mg/mTeSR) and Vitronectin/E8 (v/E8) conditions, respectively. (B) Morphology of human fibroblasts and induced pluripotent stem cells (iPSC) on days 4, 14, 22, and 28 after re-programming and grown under Matrigel/mTeSR1 (mg/mTeSR) or Vitronectin/E8 (v/E8) conditions, respectively. Arrows indicate forming iPSC colonies. Scale bar 100  $\mu$ m.

alkaline phosphatase (Figures 2A and S2) and Tra-1-60 (Figures 2B and S2). Investigation of the entire wells stained for alkaline phosphatase activity, confirmed that the colonies on the Matrigel/mTeSR1 plates were larger than the colonies

on the Vitronectin/E8 plates (Figures 2C and S2). Furthermore, the number of colonies on the Matrigel/mTeSR1 plates was significantly higher than the number of colonies grown under Vitronectin/E8 conditions, regardless of



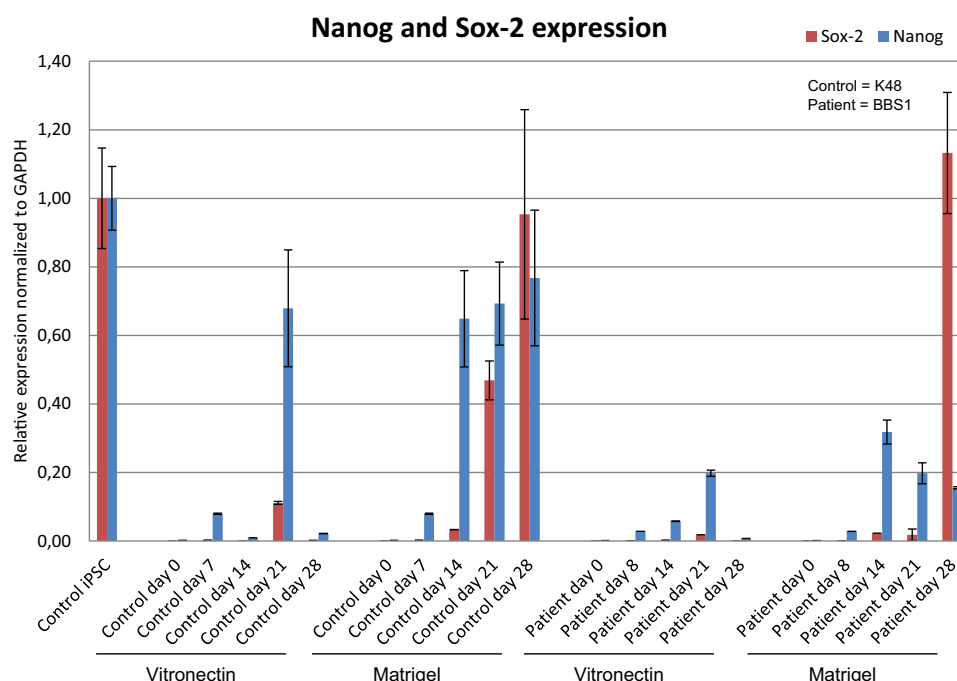


**Figure 2** Characterization of the iPSC derived from healthy control or patient fibroblasts day 28 after re-programming, grown under Matrigel/mTeSR1 (mg/mTeSR), or Vitronectin/E8 (v/E8) conditions. (A) Alkaline phosphatase staining of representative iPSC colonies. (B) Representative iPSC colonies investigated by phase contrast images and immunostained for the pluripotency marker Tra-1-60 (green). (A and B) Scale bar 100  $\mu$ m. (C) Alkaline Phosphatase (AP) staining (brown) of whole wells. Scale bar 5 mm.

whether the cells were obtained from a healthy control or a patient with Bardet–Biedl syndrome (Figures 2C and S2). The number of colonies on the Vitronectin/E8 plates for the two setups were  $36 \pm 8$  for K40/K48 and  $17 \pm 11$  for BBS1, whereas the number of colonies on the Matrigel/mTeSR plates were  $56 \pm 5$  for K40/K48 and  $25 \pm 10$  for BBS1 (Figures 2C and S2). However, because the colonies on the Vitronectin/E8 plates were very small, the total number of colonies on these plates might be underestimated.

To monitor the re-programming process, RNA from a fraction of the cells was harvested on days 0, 6–7, 14, 21, and 28 post electroporation, and analyzed by quantitative real-time PCR (RT-q-PCR) to see whether expression of the pluripotency-associated markers *NANOG* and *SOX2*

increased during re-programming. Both genes should be upregulated at the end of re-programming (Buganim *et al.*, 2012). The expression of *NANOG* and *SOX2* in cells obtained from both controls and BBS patient grown in the Matrigel/mTeSR combination had indeed increased from Day 0, whereas the expression was low/barely detectable in cells grown under the Vitronectin/E8 condition (Figures 3 and S3). The more robust signal of *SOX2* and *NANOG* in the cells grown under Matrigel/mTeSR1 conditions compared with cells grown under Vitronectin/E8 conditions might be due to a larger proportion of iPSC versus fibroblasts in the Matrigel/mTeSR1 wells compared with the cells in the Vitronectin/E8 wells, on which there was a rapid growth of non-stem cells.



**Figure 3** Monitoring of *NANOG* and *SOX2* expression during the course of re-programming. Samples are normalized to GAPDH expression and to control iPSC. Error bars represent S.E.M.

Products that contain animal derivatives, barricade any effort to achieve good manufacturing practice (GMP) standards (Lu *et al.*, 2014). Thus the specific composition of mTeSR1 and Matrigel differs from batch to batch, which makes it hard to reproduce experiments precisely. Furthermore, exposure to products of animal origin increases the risk of transmission of non-human pathogens. Thus, synthetic products are to be preferred for the generation of iPSC for clinical applications. Since colonies were formed on Vitronectin/E8 plates, this completely synthetic condition supports the growth and formation of iPSC, but must be optimized in order to obtain the isolation of larger colonies.

## Conclusions

In summary, the combination Matrigel/mTeSR is a good choice for making iPSC for research use. We found that derivation of early hiPSC of recent re-programmed fibroblasts resulted in larger and a significantly higher number of iPSC colonies, when Matrigel was combined with mTeSR1 as compared to E8 combined with Vitronectin.

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## Authors' contribution

LBM conceived the study. CABH and KBS performed all the experiments. CABH, HCB, and LBM discussed the results and prepared the first draft of the manuscript. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

The study was approved by the regional scientific ethical committee in the Capital Region of Denmark (H-3-2014-140). Written informed consent was obtained from the patients with Bardet-Biedl syndrome. The fibroblast-lines from healthy controls were anonymized.

## Conflicts of interest

The authors declare that they have no competing interests.

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## Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

**Figure S1.** Morphology of human fibroblasts and induced pluripotent stem cells (iPSC) on days 4, 10, 23, and 27 after reprogramming with the episomal plasmids expressing *hOTC4*, *hSOX2*, *hKLF4*, *hI-MYC*, *hLIN28*, and *shp53* grown under Matrigel/mTeSR1 (mg/mTeSR) and Vitronectin/E8 (v/E8) conditions, respectively. Arrows indicate forming iPSC colonies. Scale bar 100 µm. Patient: BBS1; Control: K40.

**Figure S2.** Characterization of the iPSC derived from control or patient fibroblasts, day 28 after re-programming grown under Matrigel/mTeSR1 (mg/mTeSR) or Vitronectin/E8 (v/E8) conditions. Left: Entire wells stained for Alkaline phosphatase (AP) (brown). Petri dish diameter is 3.7 cm. Middle: Selected colonies stained for Alkaline phosphatase (AP) (brown). Right: Selected colonies stained for Tra-1-60 (green).

**Figure S3.** Monitoring of *NANOG* and *SOX-2* expression during the course of re-programming. Samples are normalized to *GAPDH* expression. Error bars represent the standard deviation. Patient: BBS1; Control: K40.